NISIN INDUCED MORPHOLOGICAL CHANGES AND DISRUPTION OF GROWTH IN ESCHERICIA.COLI

DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT
FOR THE DEGREE OF
MASTER OF SCIENCE IN LIFE SCIENCE

BY
CHANDRA SWETA
413LS2052

Under the guidance of
Dr. MOHAMMED SALEEM

DEPARTMENT OF LIFE SCIENCE
NATIONAL INSTITUTE OF TECHNOLOGY
ROURKELA-769008, ODISHA

DECLARATION OF CANDIDATE

The work embodied in this report is an original investigation carried out by me, on the topic “Nisin induced morphological changes and disruption of growth in E.coli”, for partial fulfillment of degree in master of Life Science, NIT Rourkela. No part of this thesis has been submitted to any other University or Institution to confer any Degree or Diploma.

Date:

Chandra Sweta
413ls2052

राष्ट्रीय प्रौद्योगिकी संस्थान
NATIONAL INSTITUTE OF TECHNOLOGY
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RIGHT TO INFORMATION

CERTIFICATE

This is to certify that the thesis entitled "**Investigation of antimicrobial potential of Nisin against *Escherichia coli***" which is being submitted by **Ms Chandra Sweta**, Roll No. **413LS2052**, for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bona fide research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

11/5/15

Dr Mohammed Saleem
Assistant Professor
Department of Life Science
National Institute of Technology
Rourkela – 769008, Odisha, India
Phone no: 0661-2462781

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ABSTRACT:

Nisin is a 34 residue long cationic lanthionine antibiotic produced by *Lactococcus lactis* and antimicrobial activity against a broad spectrum of gram-positive bacteria. During its antimicrobial action it is known to target intermediates in the bacterial cell wall biosynthesis, lipid II, and undecaprenyl pyrophosphate. Recent discoveries of lipid II as a target for nisin has brought nisin to the forefront, as a model case, in the battle against antibiotic resistance and assessing the combination of using conventional antibiotics with nisin remain to be explored. Here we evaluated the effect of antimicrobial activity of Nisin on *E.coli* by determining the MIC (Minimum inhibitory concentration), MBC (Minimum bactericidal concentration), Zeta potential (electrokinetic potential), SEM (Scanning electron microscopy), in the presence and absence of Ampicillin. We observe that increasing concentrations of Nisin drastically prolong the lag phase of *E.coli* and cause excessive delay in reaching the stationary phase.Nisin is highly active against gram positive bacteria, but it is quite pleasing finding that it is also effective in the case of E.coli which is a gram negative bacteria.

KEYWORDS:

Antibiotics, Ampicillin, Antimicrobial peptide, Nisin, Growth curve, CFU, Electron microscopy, Membrane potential etc.

1. INTRODUCTION:

All organisms want to stay fit and healthy and for this they need protection against microorganisms and their ability to prevent the onset of infection is believed to depend on their innate immune system. However, most of the time our innate immune system fail to protect us and acquired immune system comes into the picture. Antibiotics are one of such medications, also known by antibacterials, that kill or inhibit the growth of bacteria. The Greek word anti means "against", and the Greek word bios means "life" (bacteria are life forms).

1.1 ANTIBIOTICS:

According to the US National Library of Medicine, antibiotics are a class of powerful drugs that have the potential to fight microbial infections and save lives when used properly. Antibiotics either inhibit bacteria from reproducing or completely destroy them. Overuse of

antibiotics is one of the major factors that resulted in growing number of pathogenic infections, eventually evolving resistance to antimicrobial medications. Hunt for development of novel antibiotics that could overcome resistance has thus become important due to the emergence of resistant bacterial strains worldwide (Bonomo, 2000). It has been observed that during the past two decades, living organisms of all types have become more efficient in producing a significantly large number of antimicrobial peptides. This plays an important role in innate immunity against microbial invasion. They are known to be mainly produced on epithelial surfaces as well as in phagocytic cells that play a critical role in the innate and adaptive defense systems (Ganz and Lehrer, 1995; Simmaco et al., 1998; Hancock and Scott, 2000; Yang et al., 2001).

1.2 NEED OF ANTIMICROBIAL PEPTIDE:

Antibiotic resistant bacteria are bacteria that developed some defence strategy and become powerful and are not easily inhibited or destroyed by antibiotics. They have developed the ability to survive, grow and multiply even in the presence of an antibiotic. Most infection-causing bacteria can eventually evolve resistance to at least some antibiotics. A serious threat for the health of the mankind currently is microbes that have gained defensive mechanisms against most of the antibiotics. These microbes are known as multi-resistant organisms (MROs). To treat these multi-resistant organism (MROs), we need advance antibiotics with different mode of antimicrobial action from that mode of action for which bacteria have already developed resistance. Antimicrobial peptides are now proving themselves as alternative source to kill these bacteria.

1.2.1 Antimicrobial peptide:

Antimicrobial peptides (AMPs) are a class of low molecular weight proteins with an ability to show broad-spectrum antimicrobial activity against microbes (e.g., bacteria, viruses, and fungi). These peptides are evolutionarily conserved and are usually positively charged. They are mostly amphipathic in nature that helps them to interact at interfaces and be soluble in aqueous environments yet also enter lipid-rich membranes. In general, antimicrobial peptides are determinants of the composition of the microbiota and they function to destroy microbes and prevent infections. Antimicrobial peptides are known to act against microorganisms by means of disrupting their cell membranes. Lately, the importance in human immunity, and in health as well as disease, has recently emerged and gained importance. Antimicrobial peptides are usually 12-50 amino acids residues long and are

known to include two or more positively charged residues, mostly arginine, lysine or histidine in acidic environments and a large proportion (generally >50%) of hydrophobic residues (Papagianni 2003, Sitaram & Nagaraj 2002). Hundreds of antimicrobial peptides have been known to be isolated. It is generally accepted that irrespective of the origin, spectrum of activity, and structure of the peptides, most of them share several common properties. However their effect on different bacteria varies. The interaction of most antimicrobial peptides with membranes, involving electrostatic and hydrophobic interactions, is a necessary precursor to cell death.

1.3 MODE OF ACTION OF ANTIMICROBIAL PEPTIDE

One mode of interaction of antimicrobial peptides is known to bind to negatively charged surface of membranes and permeate them, resulting in the leakage of ions and solutes (McElhaney and membranes and, 1999). However, before interacting with the phospholipid membrane peptides must pass through the negatively charged outer wall of Gram-negative bacteria containing LPS or through the outer cell wall of Gram-positive bacteria containing acidic polysaccharides. This process is known as 'self-promoted uptake' with respect to Gram-negative microorganisms (Hancock, 1997). This mechanism involves the peptides is known to initially interact with the surface LPS, competitively displacing the divalent polyanionic cations, thereby, partly neutralizing LPS. This leads to disruption of the integrity of the outer membrane and peptides thus pass through the disrupted outer membrane thereby gaining entry into the negatively charged phospholipid cytoplasmic membrane. Model membranes have been used to investigate the membrane-active properties of such peptides have been extensively studied using (McElhaney and Prenner, 1999). The amphipathicity of the peptides allows them to efficiently partition into the hydrophobic core of the cytoplasmic membrane through hydrophobic and electrostatic interactions, causing stress in the lipid bilayer. The stress developed eventually leads to the generation of unfavorable energy. The increase in the energy beyond a certain threshold leads to the loss of membrane barrier property, which is in general the basis of the antimicrobial action of these peptides.

1.4 BIOLOGICAL ACTIVITY OF ANTIMICROBIAL PEPTIDES:

A broad spectrum of activity is shown by the antimicrobial peptides. They not only kill bacteria, they are cytotoxic also to fungi (Fehlbaum et al., 1996;Kieffer et al., 2003), protozoa (Arrighi et al., 2002), malignant cells (Cruciani et al.,1991; Baker et al., 1993; Lindholm et

al., 2002), and even enveloped viruses like HIV, herpes simplex virus, and vesicular stomatitis virus (Tamamura et al., 1998;Robinson et al., 1998). Different microorganisms have different possession of a distinct cell membrane that defines characteristics of the antimicrobial peptides. On the one hand, many antimicrobial peptides display broad-spectrum activity against Gram-negative bacteria, Gram-positive bacteria, and fungi (Miyasaki and Lehrer, 1998). The molecular basis of the selectivity of the antimicrobial activity of the peptide is not completely understood.

1.4.1 Antimicrobial Peptides and its diversity:

Nearly about five hundred antimicrobial peptides have been discovered so far.They are unique in respect of its sequence, their diversity is such that the same peptide is very rarely rcan be obtained from two different species of living species. We can classify the antimicrobial peptides on the basis of different criteria and one of the criteria is its secondary structure. (EpandandVogel, 1999; van't Hof et al., 2001). The fundamental structural principle is the capacity of a peptide to have a shape in which clumps of hydrophobic and cationic amino acids are spatially organized in distinct parts of the molecule. Antimicrobial peptides are classified into four groups according to their secondary structure:(proposed by van't Hof et al. (2001)):

Group I: peptides with an α-helical structure and have linear shape;

Some the peptides like magainin, cecropin A, temporins, number of de novo designed antimicrobial peptides (Boman, 1995; Mangoni et al.,2000) have structures which is not in order in aqueous solution while fold into an α-helical conformation upon interaction with hydrophobic solvents or lipid surfaces. α-Helical peptides are maximally time found to be amphipathic and can either absorb onto the membrane surface or insert into the membrane as a aggregate of helical bundles.

Group II: restrained peptides which are confomationally intact, predominantly consisting of β-strands connected by intramolecular dioether bridges:

β-sheet peptides are cyclic peptides constrained either by diether bonds , as in the case of human β-defensin-2 (Hancock, 2001),that is just reverse to α-helical structured peptides or or by cyclization of the peptide backbone, as in the case of gramicidin S (Prenner et al., 1999).

They mainly prevail in the β-sheet conformation in aqueous solution that can be further stabilized upon interacting with lipid surfaces.

Group III: peptides with an extended structure and have linear structure, characterized by over representation of one or more amino acids:

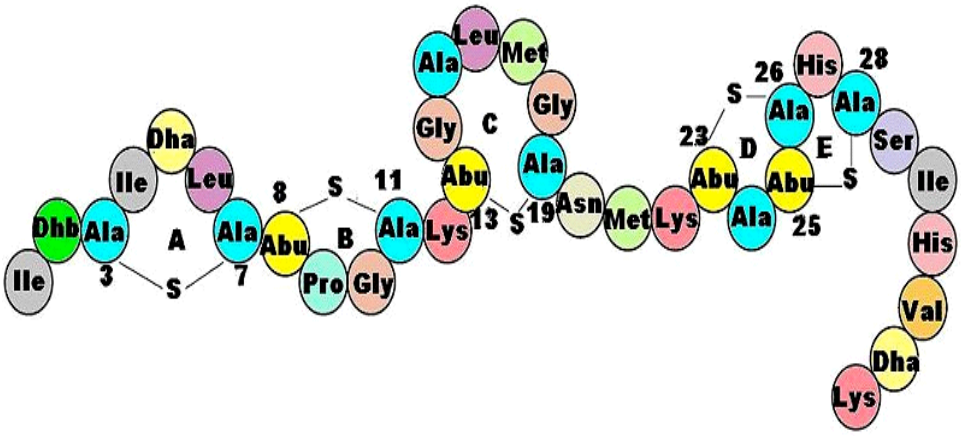
More or less, some antibacterial peptides show an unusual amino acid composition, having a sequence that contains more number of one or more specific amino acids. Lets take some exmple, the peptide histatin, which is mainly produced in animal saliva, is highly rich in His residues (table 1, Brewer et al., 1998; Tsai and Bobek, 1998; Helmerhorst et al., 1999a).

Group IV: looped structured peptides:

Peptides that are rich in proline-arginine cannot form structures are amphipathic due to the severly of high concentration of proline amino acid residues in such structures and they are proposed to havet a poly proline helical structure which is type-II structure (Boman et al., 1993; Cabiaux et al., 1994). Lantibiotics having small lring structures surronded by a disulphide bridges bond and structure and properties of them have recently been reobseved (Montville and Chen, 1998). One of the lantibiotics, nisin, iscurrently used as an antimicrobial agent for food preservation and this peptide has relatively high activity against Gram-positive bacteria due to its specific high affinity with Lipid II, a precursor in the and de Kruijff, 1999; Breukink et al., 1999).

1.5 NISIN AS ANTIMICROBIAL PEPTIDE:

Nisin which is 34-a.a residue, antibacterial peptide produced by fermentation of Lactococcuslactis that is highly active against gram-positive bacteria. The dehydrated residues which are highly present and lanthionine rings (thioether bonds) in nisin, impart structural restrains on the peptide structure, make it an interesting for studying the mode of action(EefjanBreukink, Ben de Kruij etal,1999).



Fig; 1. Schematic for structure of nisin

Nisin binding:

Nisin binds preferably to membrane containing anionic lipids that should be present in relatively large amount which is very common in Gram-positive bacteria .

Nisin insertion and orientation:

The amphipathic properties allow nisin to insert into the lipid phase of cell membrane. The anionic phospholipids are essential for efficient insertion of nisin. Nisin variants with either extensions oat N-terminus, or with minor changes in the first ring severly reduce the ability to insert in the lipid monolayer where C-terminus does take part in the insertion. Hence, it is clear that the N-terminus of the nisin insert first. It is also proved that an increase in the amount of antibiotic lipids results in the increased depth of insertion of nisin molecule. The stable orientation of nisin in the membrane is parallel with respect to the membrane surface.

Pore formation:

The amount of bound nisin greatly influences the amount of leakage that occurs. The size of the nisin pore is supposed to be 1 nm.

1.6 BACTERIA:

Bacteria makes the major section of micro-organisms. They are miniaturel, only micro- meter in length. Bacteria evolve first were suppose to first form of life, which appear on Earth and

present in almost all of its habitats. The vast majority of bacteria are harmless but some are pathogenic (opportunistic) and cause infectious diseases.

Extracellular structures of bacteria:

The cell wall is present over of the Cell membrane of the almost all bacteria. A common bacterial cell is consist of peptidoglycans, which are made from polysaccharide chains cross linked by peptides containing D-amino acids. There are two different types of cell wall in bacteria, a thick one is the gram- positives and a thinner one in the gram-negatives.

- GRAM POSITIVE BACTERIA: These bacteria have a thick cell wall containing teichoic acids and many layers of peptidoglycan.
- GRAM NEGATIVE BACTERIA: In bacteria, gram contrast to gram positive negative bacteria have a relatively thin cell wall consisting of layers of peptidoglycan surrounded by second membrane consisting lipo-polysaccharides and lipo-protein. Lipo- polysaccharides, also called endotoxins, are composed of polysaccharides and lipid A that is responsible for making gram-negative bacteria more toxic.

1.6.1 ESCHERICHIA COLI:

Escherichia coli is commonly abbreviated as E. coli. It is gram negative , facultatively anaerobic and rod- shaped bacteria. It is commonly found in the lower intestine of warm – blooded organisms. Most of the E.coli strains are harmless, but some are serotypes which can cause serious food poisoning. Some virulent strains can cause gastroenteritis, urinary tract infections, and neo –natal meningitis. In some cases, virulent strains can cause hemolytic uremic syndrome, peritonitis, mastitic, septicemia and gram negative pneumonia.

Some strains such as E.coli0157:H7, can cause severe abdominal cramps, bloody diarrhorea and vomiting. Humans are easily exposed to E.coli from contaminated water or food especially raw vegetables and undercooked non veg food.

1.7 AMPICILLIN (ANTIBIOTIC USED):

Ampicillin does not show severe side effect and commonly used antibiotics.

Ampicillin is an antibiotic useful for the treatment of a number of bacterial infections and can be used against both gram positive and gram negative bacteria. It is a beta-lactam antibiotic that is part of the aminopenicillin family and is roughly equivalent to amoxicillin in terms of

activity (American Society of Health-System Pharmacists-2006). It works by killing sensitive bacteria by interfering with formation of the bacteria's cell wall while it is growing. This weakens the cell wall and it ruptures, resulting in the death of the bacteria. It can be used for the treatment of urinary tract infections, meningitis, and salmonella infections, but resistance to ampicillin is increasingly common among the bacteria responsible for these infections like E.coli is getting resistance over ampicillin. So effect of this antibiotic with combination of nisin with different concentration is carried out, and found some interesting fact that the effectiveness of ampicillin is increasing with the combination of antimicrobial peptide nisin.

2.METHODS AND METHODOLOGY:

Different experiments are carried out to get the idea about how the nisin affect the growth of bacteria (E. coli) and simultaneously how the nisin can increase the effectiveness of ampicillin. Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), SURFACE POTENTIAL MEASUREMENT, EM, CELL VIABILITY Experiments were done.

2.1 MIC (MINIMUM INHIBITORY CONCENTRATION):

Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation.

Media Preparation:

13g of nutrient broth should dissolve in 1000ml of water. We had to make 75ml of the solution. So the nutrient broth required is $13 \div 1000 \times 75 = 0.975$g.

- 0.975g of nutrient broth was weighed.
- It was dissolved in 75ml of distilled water in a measuring beaker.
- Three measuring conical flasks were taken.
- 25ml of media was poured in each flask.

- The flasks should be tightly plugged with cotton plugs.
- The flasks were wrapped with paper.
- They are autoclaved for 15 minutes.
- The media was ready to use.

*1mg/ml antibiotic stock solution was prepared.

*To calculate the amount of Nisin stock solution required, to do MIC test, we can use this formula:

$C_1V_1=C_2V_2$

Where, V_1=?

C_2=50U/ml

V_2=300µl

Hence, V_1=15µl

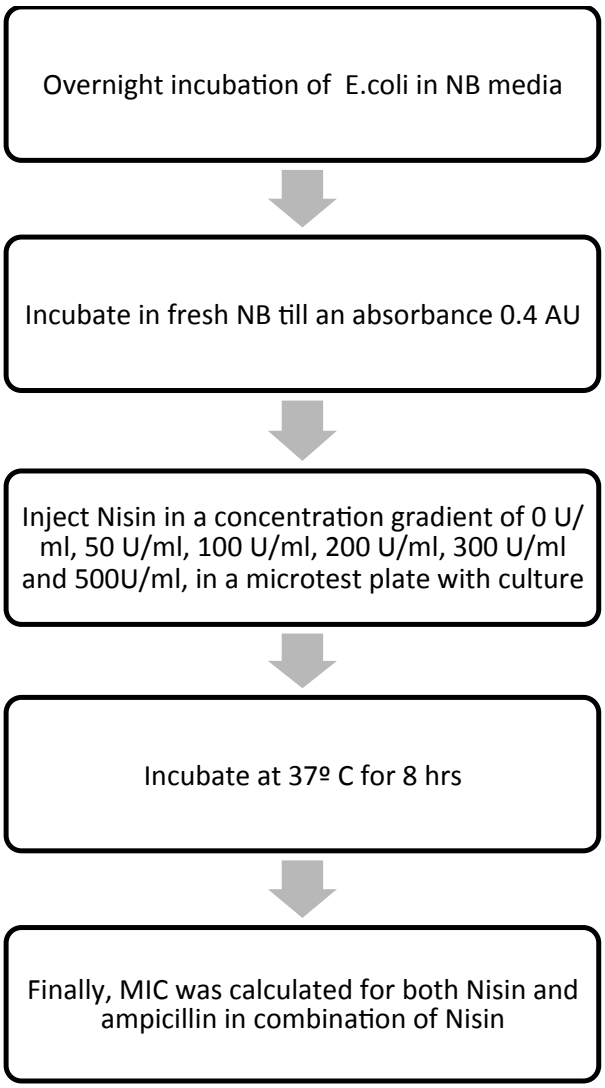
C_1=1000U/ml.

Tabulation for MIC (Well-Plate preparation):

Sl no.	**Solution (U/ml)**	**Nisin(µl)**	**Culture(µl)**	**Ampicilin(µl)**
1	Control	0	30	0
2	50	15	30	0
3	200	60	30	0
4	500	150	30	0
5	1000	60	30	0
6	1500	90	30	0
7	Amp-Control	0	30	30
8	50	15	30	30
9	200	60	30	30
10	500	150	30	30
11	1000	60	30	30
12	1500	90	30	30

PROTOCOL FOR MIC EXPERIMENT:

To carry out MIC Experiment, following steps should be followed carefully. NB media has been used.

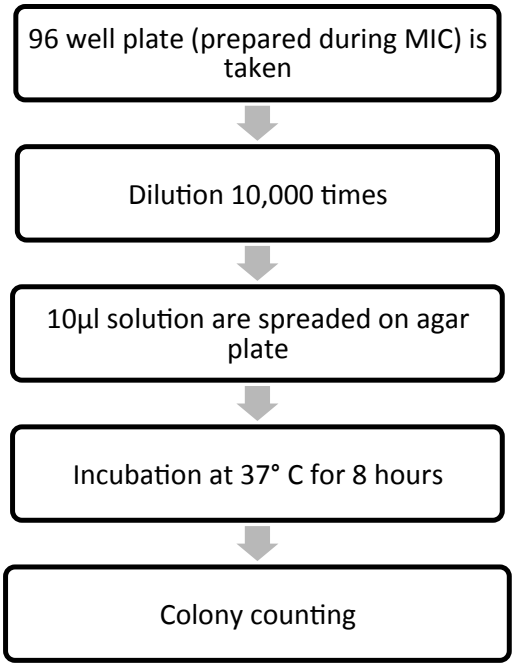


2.2 Minimum Bactericidal Concentration (MBC) Test:

The MBC test determines the lowest concentration at which an antimicrobial agent will kill a particular microorganism. The MBC is determined using a series of steps, undertaken after a Minimum Inhibitory Concentration (MIC) test has been completed. It can be determined

from broth dilution minimum inhibitory concentration (MIC) tests by subculturing to agar plates that do not contain the test agent. The MBC is identified by determining the lowest bacterial inoculum by ≥99.9%. Antibacterial agents are usually regarded as bactericidal if the MBC is no more than four times the MIC.

Protocol used:



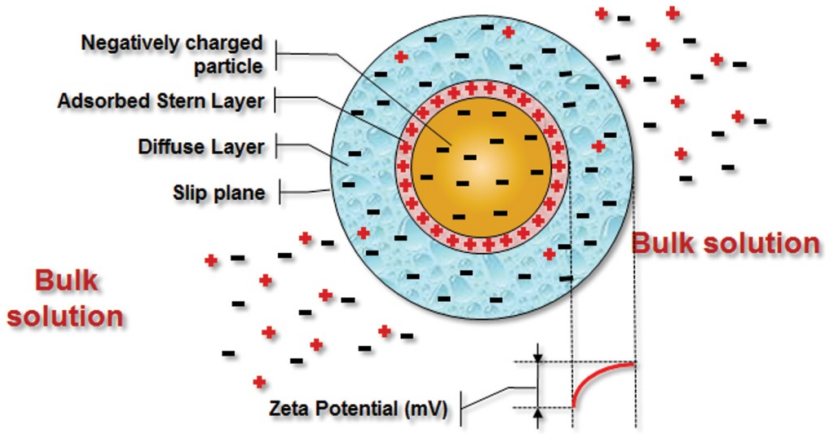
2.3 CFU (Colony Forming Unit):

In microbiology, a colony-forming unit (CFU) is a unit used to estimate the number of viable bacteria or fungal cells in a sample that has the ability to multiply by binary fission under the controlled conditions. Checking with CFU obliges refined the microorganisms and checks just feasible cells, interestingly with infinitesimal examination which numbers all cells, living or dead.

2.4 SURFACE POTENTIAL TEST (ZETA POTENTIAL MEASUREMENT):

Zeta potential is a scientific term for electro-kinetic potential in colloidal dispersions (Definition of electro-kinetic potential in "IUPAC. Compendium of Chemical Terminology", 2nd ed. (the "Gold Book"). Zeta potential is the potential difference between the dispersion

medium and the stationary layer of fluid attached to the dispersed particle. The zeta potential is caused by the net electrical charge contained within the region bounded by the slipping plane, and also depends on the location of that plane. Thus it is widely used for quantification of the magnitude of the charge. The zeta potential is a key indicator of the stability of colloidal dispersions.



Schematic representation of Zeta potential Wikipedia.org

Measurement of zeta potential:

Zeta potential is not measurable directly but it can be calculated using theoretical models and an experimentally-determined electrophoretic mobility or dynamic electrophoretic mobility.

Ideal samples from Zeta Potential Analysis are:

- Samples should be in monodisperse in size.
- Are at a high enough concentration to effectively scatter 633 nm light
- There should be very low salt concentrations (conductivities <1 mS/cm)
- Are suspended in a particulate free, polar dispersant (e.g. high purity water).

Protocol used:

Preparation of HEPES buffer:

HEPES-10mM (molecular weight- 238.30g/mol)

NaCl-150mM(molecular weight- 58g/mol)

pH- 7.4

Volume-500ml

HEPES:

W= m.w×M×V÷1000

=238.30×10×10-3×500÷1000

=1.1915g

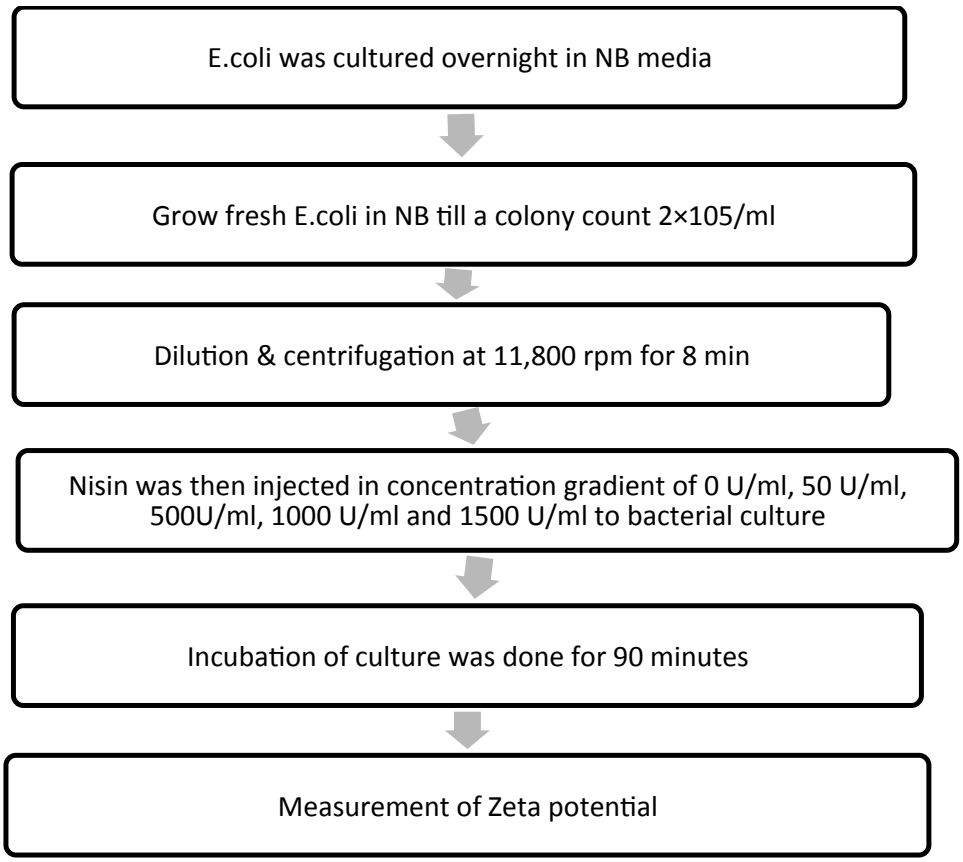
NaCl:

W=58.5×150×10-3×500÷1000

=4.38g Hence 1.1915g of HEPES buffer and 4.38g of NaCl were dissolved in milli Q (deionised) water.The volume was made upto 500ml. The pH is maintained at 7.4.

Flow chart of steps during zeta potential measurement are described below.

Dilution of the sample should be done in hepes buffer.



2.5 SCANNING ELECTRON MICROSCOPY (SEM):

The SEM instrument is made up of two main components or parts, the first one is electronic console and the second important component is the electron column. The electronic console provides control knobs and switches that allow for instrument adjustments such as filament current, accelerating voltage, focus, magnification, brightness and contrast (Taken from Introduction to Scanning Electron Microscopy ,By: Brandon Cheney).

ELECTRON COLUMN:

The electron column is the place, where the electron beam is generated under vacuum the condition, focused to a small diameter, and scanned across the surface of a specimen or sample by electromagnetic deflection coils. The lower portion of the column is called the specimen chamber or sample chamber. The secondary electron detector is located above the sample stage inside the specimen chamber. Specimens are mounted and kept secured onto the stage which is controlled by a goniometer.

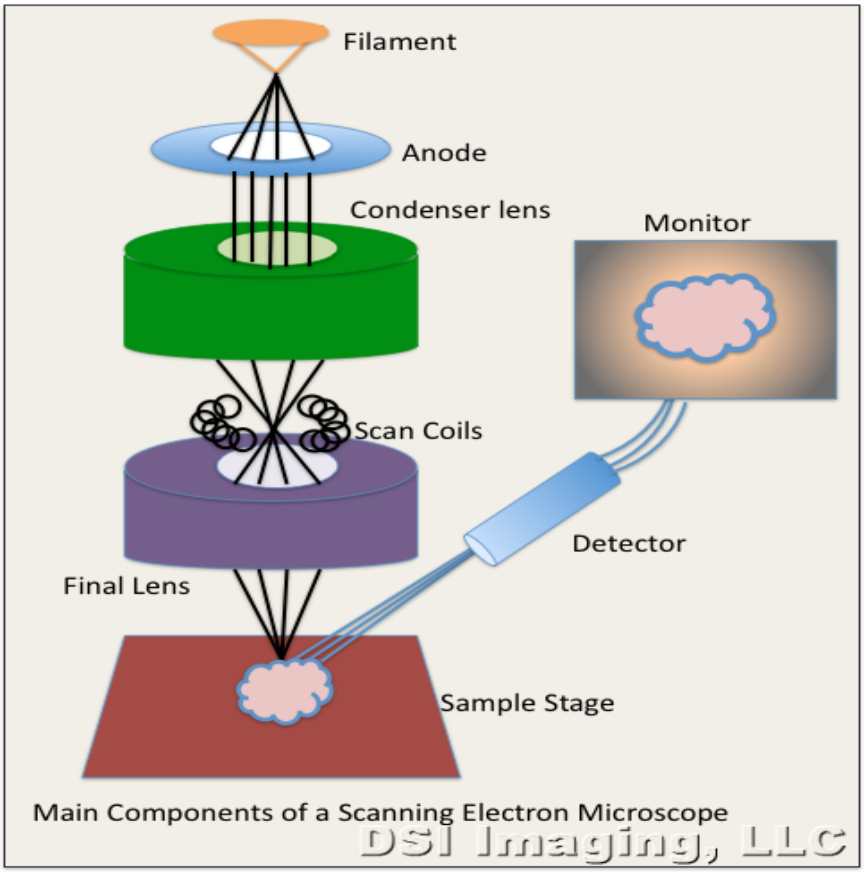


Fig:2. Scanning electron microscope column (taken from Introduction to Scanning Electron Microscopy, DSI, Imaging, LLC).

Preparation of specimen:

For SEM & FESEM, samples are fixed in glutaraldehyde, dehydrated through a series of solvents and dried completely either by air or by critical point drying. The specimens are then mounted on a special metal holder or stub and coated with a thin layer of gold or platinum before viewing in the EM.

Slide preparation protocol (SEM):

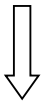
1.5ml of overnight culture was taken

⇩

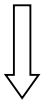
Centrifuge at 5000 rpm for 5min.

⇩

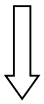
Pellets were collected and washed twice with PBS by centrifugation at 5000rpm for 5min.



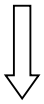
Pellets were resuspended in PBS



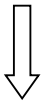
One drop of resuspended pallet was taken on the glass slide



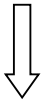
The glass slides were flooded by gluta aldehyde (2.5%) prepared in PBS



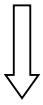
The slides were kept for overnight (about 15 hr) incubation at 4^{o}c



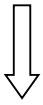
Wash with water



11% tannic acid was flooded over slides



Kept for 5 minutes



Wash with distilled water

The slides were dehydrated with ethanol sequentially

30% 50% ~~70%~~ ~~90%~~ ~~100%~~

Slides were kept for drying

2.6 BacLight Fluorescence Cell Viability Assay:

LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen) was used to determine the bacterial cell viability. Conventional direct-count assays of bacteria viability are based on metabolic characteristics or membrane integrity.Cells with a compromised membrane that are considered to be dead or dying will stain red, whereas cells with an intact membrane will stain green.

It is compatible for bacterial cells and it detects images by fluorescence detection method. The commercially available LIVE/DEAD BacLight kit (Invitrogen) consists of two stains, propidium iodide (PI) and SYTO9, which both stain nucleic acids. Green fluorescing SYTO9 is able to enter all cells and is used for assessing total cell counts, whereas red fluorescing PI enters only cells with damaged cytoplasmic membranes. The emission properties of the stain mixture bound to DNA change due to the displacement of one stain by the other and quenching by fluorescence resonance energy transfer. Although this kit enables differentiation only between bacteria with intact and damaged cytoplasmic membranes, it is often used to differentiate between active and dead cells. While it seems accurate to assume that membrane-compromised bacterial cells can be considered dead, the reverse (that intact cells are active cells) is not necessarily true. Microscopic assessment of LIVE/DEAD-stained bacterial cells is usually simplified to either “green”-labeled (live) or “red”- labeled (dead) cells.

3. OBSERVATION AND RESULTS:

3.1 MINIMUM INHIBITORY CONCENTRATION:

96 well plates were made and OD reading was recorded for every half an hour and graph is plot.

MIC: (for 6 hours)

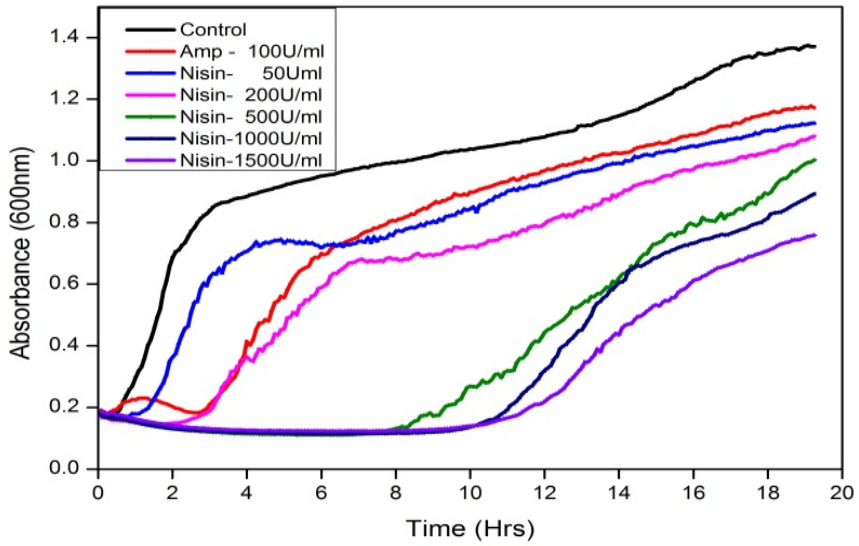


Figure. 3. Showing effect of only nisin on bacterial growth in time vs absorbance (OD) in 6 hrs.

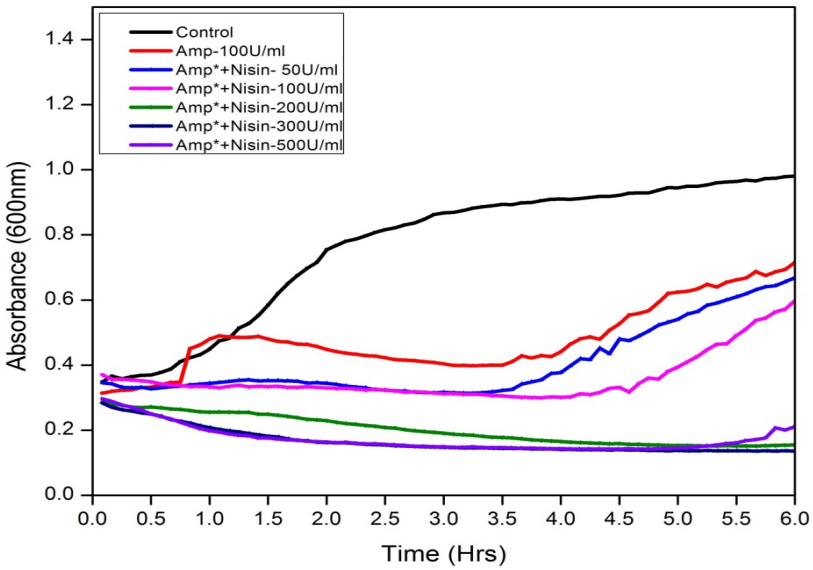


Figure. 4 Showing effect of nisin with ampicillin on bacterial growth in time vs absorbance (OD) in 6 hours.

MIC (for 20hours):

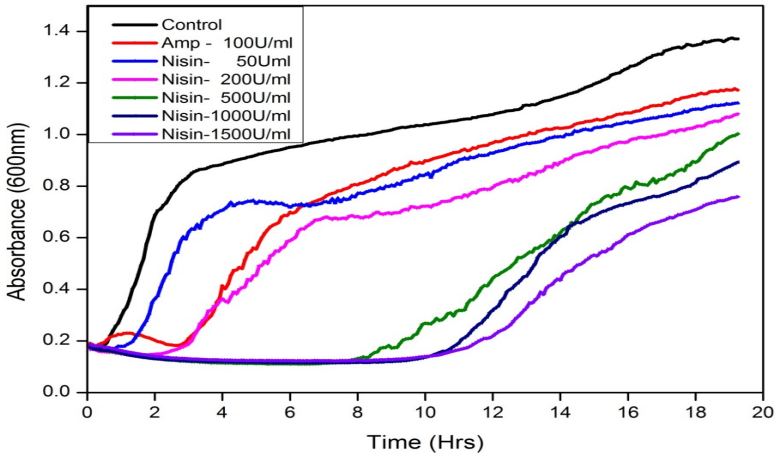


Figure. 5.Showing effect of only nisin on bacterial growth in time vs absorbance (OD) in 20 hours.

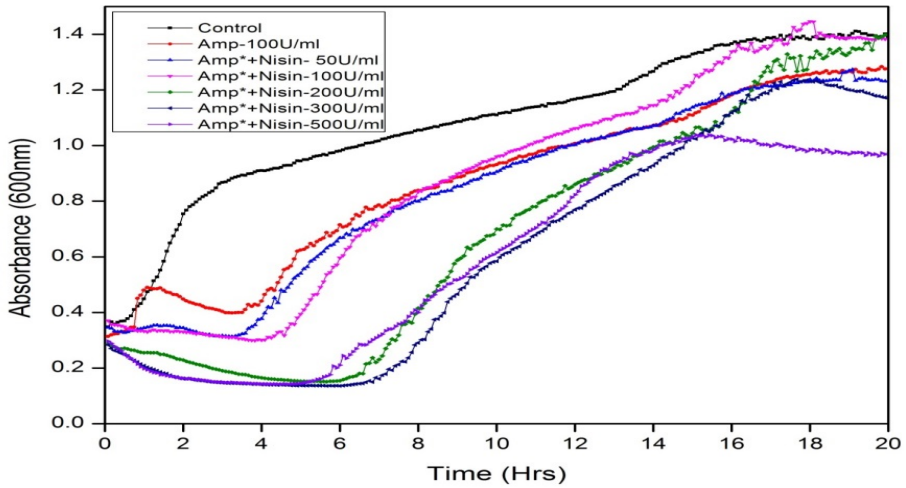


Figure. 6 Showing effect of nisin with ampicillin on bacterial growth in time vs absorbance (OD) in 20 hours.

Above graphs show prolongation of lag phase of bacterial growth in the presence of nisin and the same condition is observed when nisin is applied with ampicillin.

As the concentration of nisin increases, number of bacterial colonies decreases and the same result is observed in case of nisin with ampicillin.

3.2 CFU:

TABLE1: CFU/ml as in increasing order of nisin concentration.

Sl.no.	Nisin(in U)	CFU/ml	Log_{10}CFU/ml
1.	0	8.4×10^7	7.9242
2.	500	8.2×10^7	7.9138
3.	1000	4×10^7	7.6020
4.	1500	3×10^6	6.4771

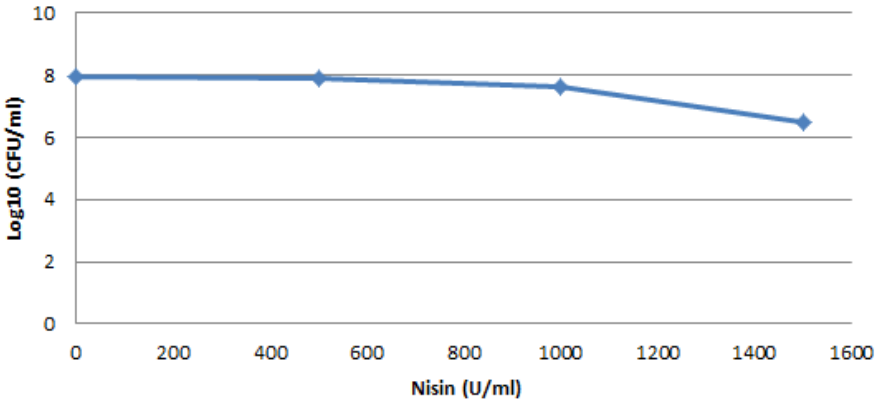


Figure. 7. Graph showing decreasing of log_{10}CFU/ml as nisin concentration is increasing.

Sl.No.	Amp. + Nisin conc.U/ml	CFU/ml	Log_{10}CFU/ml
1.	0	$11.8*10^7$	8.0718
2.	50	$5.4*10^7$	7.7323
3.	500	$3.2*10^7$	7.5051

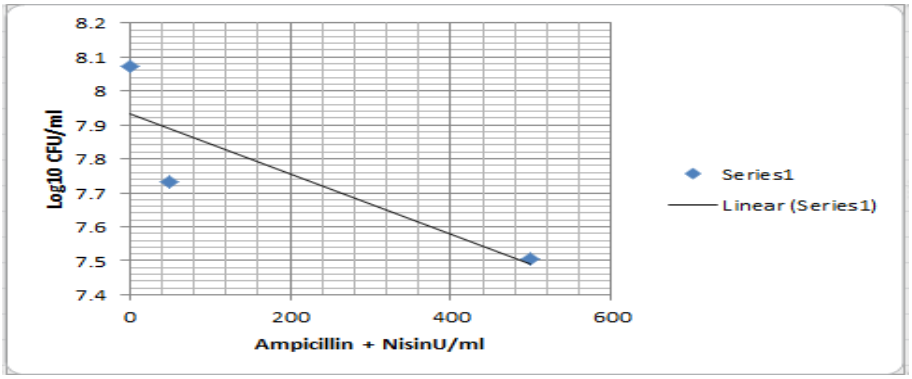


Figure. 8. Graph showing decreasing of log_{10}CFU/ml as nisin concentration is increasing.

3.3 Zeta Potential (Surface charge potential):

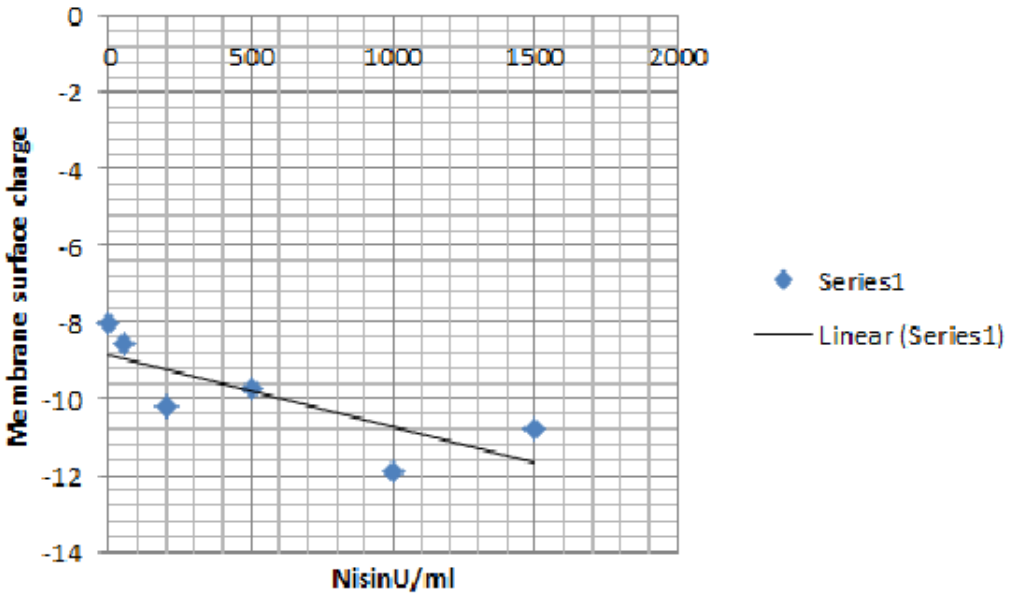


Figure. 9. Graph showing surface charge during different concentration of nisin

From the above graph we observed that, increasing concentrations of nisin shows somehow increase in negative surface charge of *E.coli* .

3.4 Scanning Electron Microscopy (SEM): (at 10,000 magnification):

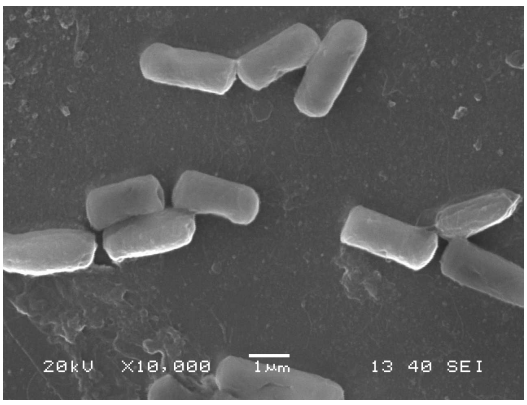


Figure. 10. *E.coli*

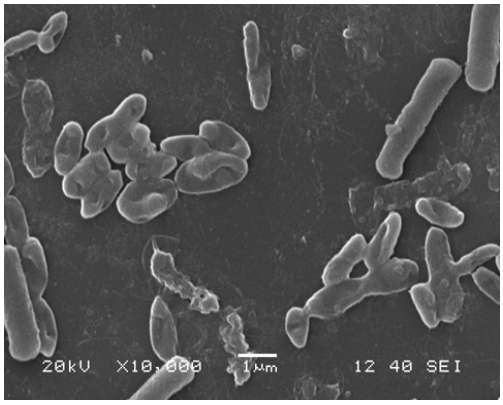
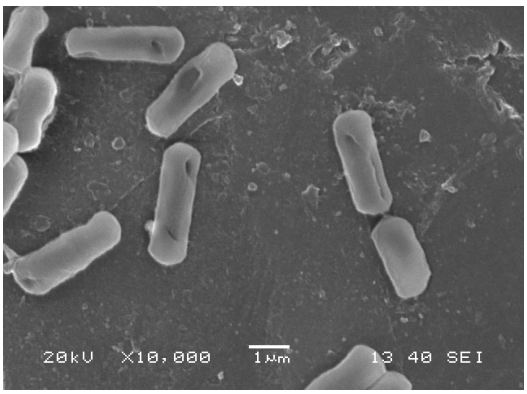


Figure. 11. *E.coli* with 1500 U/ml nisin



Figure;12. *E.coli* with ampicillin.

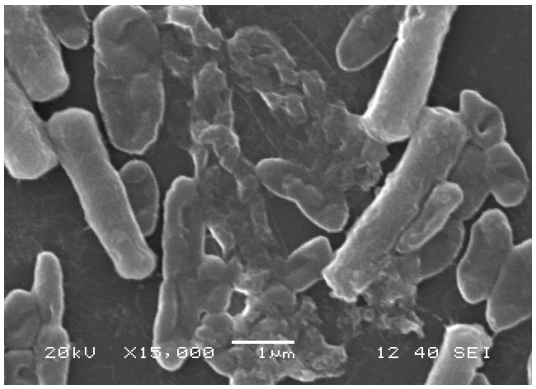


Figure. 13. *E.coli* with amp. + 1500 U/ml nisin

Fig:10, shows normal e.coli bacteria. Fig:11, shows that with the increasing concentration of nisin the structure of the cells get deformed. In fig;12, the cells are normal but in case of fig:13, where nisin is applied with ampicillin, pore formation in the cells are visible.

TABLE3:

Tabulation (measuring the length, breadth, radius and volume):

S.No	***Image***	**Length (L) (µm)**	**Breadth (w) (µm)**	**Radius (R) (µm)**	**Volume ($µm^3$)**
1	Control	2.91±0.082	1.01±0.05	5.94×10^{-3}	3.189
2	Nisin 50U/ml	2.29±0.041	0.82±0.91	3.72×10^{-3}	1.372
3	Nisin 1500U/ml	1.89±0.18	0.***49***±*0*.82	1.56×10^{-3}	1.335
4	Amp Control	2.42±0.091	0.76±0.94	5.70×10^{-3}	2.241
5	Amp+Nisin1500U/ml	1.8+0.12	0.48±0.08	1.21×10^{-3}	1.109

The images on the previous leaf and the tabulation above show that, there is a decrease in length, breadth, radius of the bacterial cell when the nisin concentration is increasing. While the volume is calculated from the 2D images, they are also decreasing accordingly.

The treatment of nisin leads to tapering of cell in both the sides. That would be the result from damage of cell membrane.

Cell membrane can be deformed by various reasons. Loss of phospholipids from decreased synthesis or increased degradation is an important mechanism to cell damage. As nisin reduces the production of necessary enzymes, DNA, RNA etc., that may lead to loss of phospholipids which may taper the cells.

The cytoskeleton composed of microfilaments, intermediate filaments and microtubules serves as a structural support system and transport system for the cell. Detachment of the cytoskeleton from the plasma membrane is caused by nisin intoxication which results in membrane deformation.

Scanning Electron Microscopy (At 10,000 magnification):

Rupturing or defomation of cell membrane can easily be seen by taking images in scanning electron microscopy. Thermal LUT make the clear distinction between intact cell membrane and deformed cell membrane

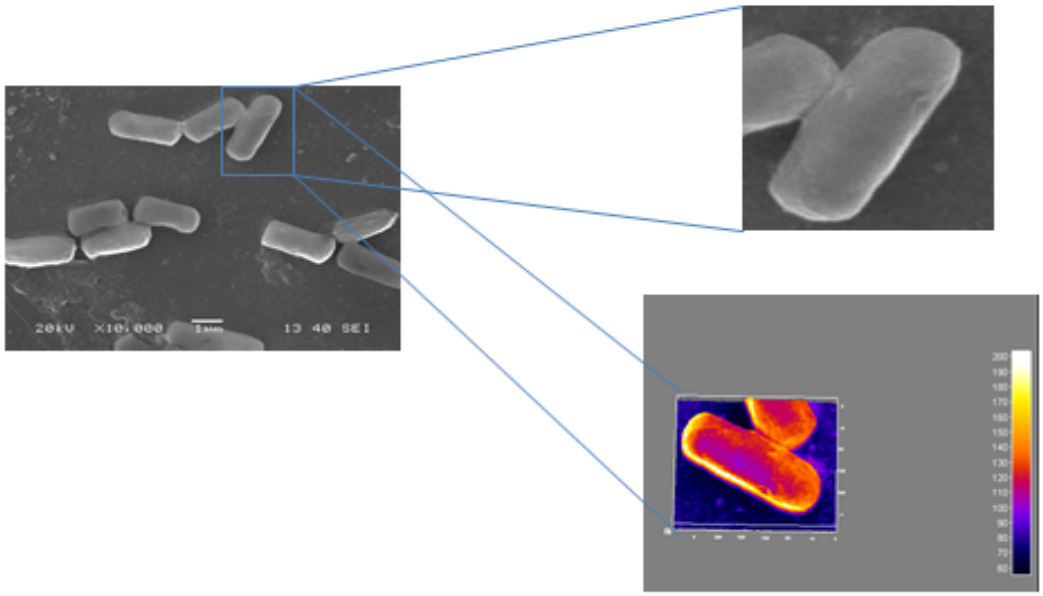


Figure. 14. The thermal LUT images above show no deformation of cell membrane when a cell is culture only in media.

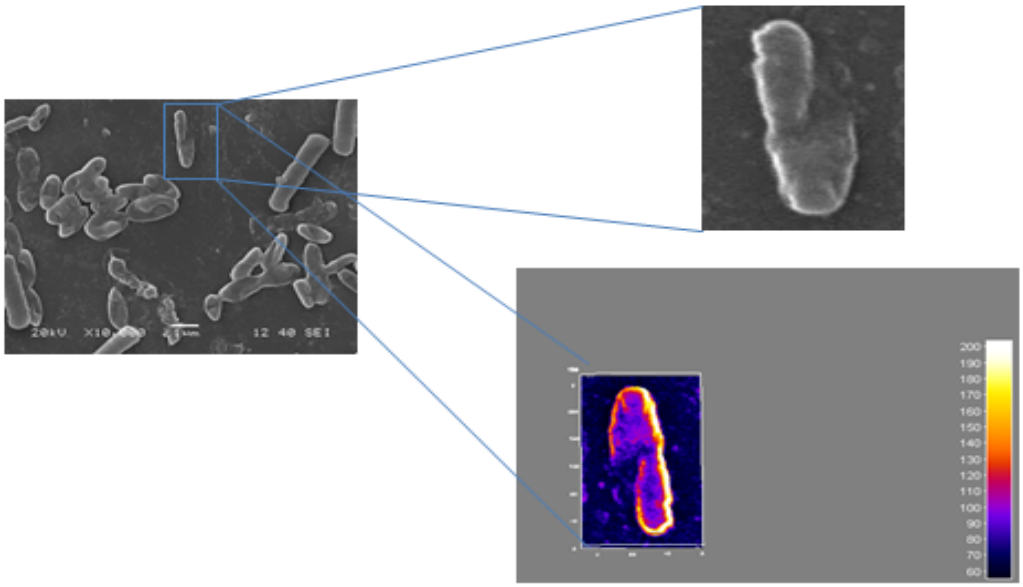


Figure. 15. The thermal LUT images above show the deformation of cell when a cell is treated with nisin (1500 U/ml).

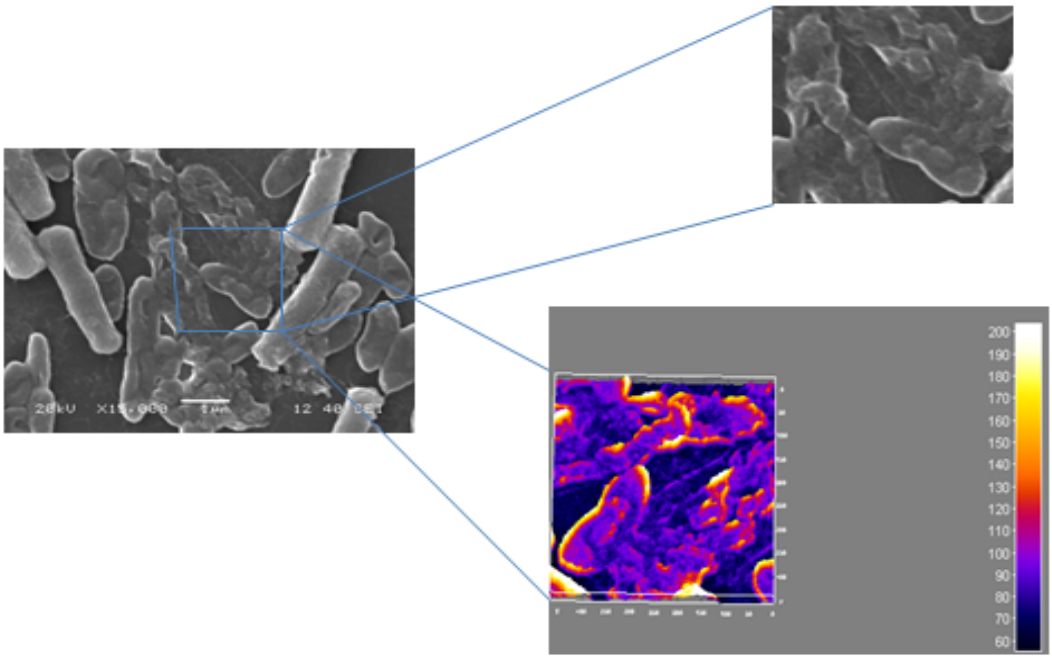


Figure. 16. The thermal LUT images above show the deformation of cell when a cell is treated with ampicillin + nisin (1500 U/ml).

BACLIGHT:

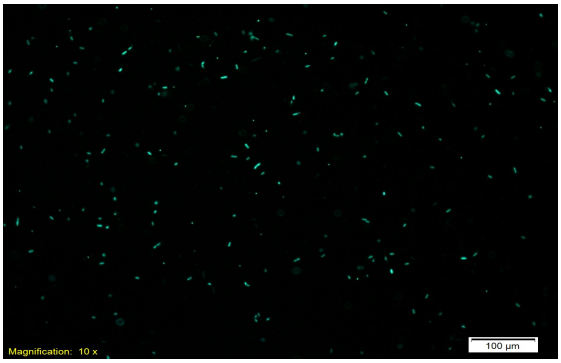


Figure. 17. E.coli control

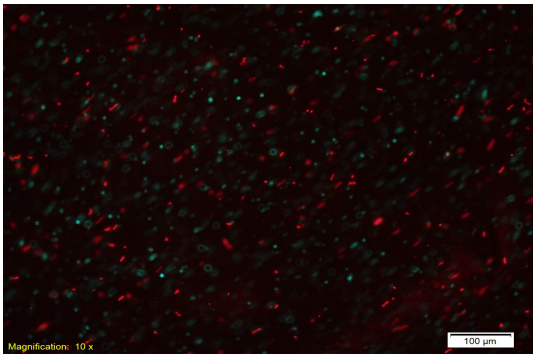


Figure. 18.E.coli with 1500U/ml nisin

The kit consists of two stains, propidium iodide (PI) and SYTO9, which both stain nucleic acids. Green fluorescing SYTO9 is able to enter all cells and is used for assessing total cell counts, whereas red fluorescing PI enters only cells with damaged cytoplasmic membranes.

The green colour in the fig: 17. shows that at nisin (control), the cells are alive, and in fig: 2, the red colour indicates most of the cells are dead due to the incorporation of nisin (1500U/ml).

4. DISCUSSION:

In the present study, we investigate the morphological changes in *E.coli*, a Gram-negative rod shaped bacteria, after incubation with nisin to analyze the effects of the nisin in the morphology of the E.coli, simultaneously we also want to check whether it has positive effect when mixed with antibiotic on the bacteria or not. And we have observed that it is more effective when it is mixed with antibiotic.

In MIC test it is observed that with increasing concentration of nisin, the prolongation of lag phase occurs. This may reflect that nisin reduces the ability of the bacterial cells to stick to the lag phase, where the cell increases its size and makes itself ready to divide by synthesizing necessary enzymes, DNA, RNA etc.

The decrease in the number of bacterial colonies in MBC test suggests, higher concentration of nisin leads to cell death.

The Zeta potential result shows some fluctuation in surface charge when concentration of nisin increases. Why there is fluctuation in surface charge during increament of nisin concentration need to be explored.

The images from electron microscopy show reduction in cell length, which indicates that cell-wall inhibition occurs mainly during the active cell elongation of bacteria division cycle. The tapering of cell may be caused due to loss of phospholipids from the cell membrane. Also the cytoskeleton may get detached from the cell membrane, so that the membrane loses its elasticity and gets tapered.

In baclight test, we have found that nisin is effective against *E.Coli.*

5.CONCLUSION:

Nisin induced leakage of cytoplasmic contents from treated samples, It seems to hinder growth of *Ecoli.* The cell division of the bacteria is drastically slowed down and the shape and size get significantly affected that changes the cylindrical cells to tapered ones.

6. FUTURE PERSPECTIVE:

E.coli is a gram negative bacteria. As the earlier findings suggest us that nisin should not be active against *E.coli,* as nisin is not effective against gram negative bacteria. However we have observed through various experiment that it is upto some extent effective against *E.coli.*

How nisin is deforming the plasma membrane, whether it is interrupting the cell division machinery and like this so many mechanisms need to be explored.

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